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Molecular characterization of human papilloma virus from women attending selected hospitals Abuja, Nigeria

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ABSTRACT

Human Papilloma Virus (HPV) infection is the leading risk factor for cervical cancer and other cancers, persistent infection leads to disease progression. About 40 HPV species have been identified to be associated with the genital mucosal, which are categorized according to their carcinogenic potential. The aim of this study was to identify the strains of HPV among women attending selected hospitals in the Federal Capital Territory, Abuja, Nigeria. The study was hospital based, where cervical swab samples were randomly collected from 501 women. Structured questionnaires were administered to the women, after an informed consent had been obtained from the women. Cervical swabs were collected from the cervix and DNA was obtained by extraction. PCR method was done using consensus primer sets MY09/MY11 and GP5+/GP6+. HPV DNA sequencing was done using Sanger Method. The HPV types identified were HPV 6 (6.67%), HPV 16 (13.33%), HPV 18 (13.33%), HPV 58 (13.33%), HPV 70 (33.33%), HPV 72 (6.67%) and HPV 81 (13.33%). The most prevalent type identified was HPV type 70. High risk type identified included HPV 16, 18 and 58.Based on the results there is a need to increase the level of surveillance on females at risk of cervical cancer in this environment, since significant proportion of highly oncogenic strains with a high tendency to transform into malignancy were observed in this study. The results from this study also contributed to the epidemiological data on the distribution of HPV within this region.

INTRODUCTION

Human Papilloma Virus (HPV) belongs to the Papillomaviridae family and it establishes infections only at the stratified epithelium of the skin or mucous membrane leading to the development of cancer [1]. Approximately 200 species of HPV have been identified and about 40 HPV species have been identified to be associated with the genital mucosal, which are categorized according to their carcinogenic potential [2]. Clinical manifestations of HPV infection includes genital warts, recurrent respiratory papillomatosis, Cervical Intraepithelial Neoplasia (CIN), and cancers, including cervical, anal, vaginal, vulva, penile, head and neck cancer [3].

Cervical cancer is the second most common malignant cancer in women and is a major cause of cancer related death among women worldwide[4, 5, 6]. The prevalence of HPV infection has been reported to be between 10-20% in Sub-Saharan Africa. A cross-sectional study has revealed high prevalence rate of infection to be between 20% - 40% among sexually active young women which decreases as they age[1].

This study will provide baseline information on the circulating genotypes of HPV in the study area. Furthermore, there are insufficient data on the phylogeny of HPV in the study area and so, the evolutionary history and extent of species mutation is unknown. The above mentioned challenges require urgent attention to reduce the mortality rate of cancer caused by HPV. This will enable the implementation of health policy interventions to control cervical cancer and other cancers associated with HPV among population under study. This study was aimed at providing information on the strains of HPV among

women attending selected hospitals in the Federal Capital Territory, Abuja, Nigeria compare the strains with those isolated from other regions and construct a phylogenetic tree to determine the level of relatedness.

MATERIALS AND METHOD

Study Population:

The hospital based study involved 501 women between March to June 2018. Structured questionnaires were administered to the women, after an informed consent had been obtained from the women. Ethical approval for this study was obtained from the Research and Ethics Committee of the federal capital territory administration and the hospitals involved in the study before the commencement of the study. Women who were attendees of the selected hospitals and they were within the age group of 15 to 65 years and had given their consent were included in the study.

Sample Collection and Preparation:

Cervical smears were collected from 501 women. Each of the participants was placed in a dorsal position, with her legs flexed at the hip and knee abducted. The labia were parted with gloved thumb and index fingers. A Cusco's bivalve speculum which is not lubricated was passed and fixed to visualize the cervix, under a bright light source. The detachable end of the cervical brush; Rovers® Cervex-Brush® cell sampling device (Rovers Medical Devices B.V 5347 KV Oss, The Netherlands) was then inserted into the cervix and rotated through 360° movements, either in a clockwise or counter clockwise direction, to scrape the entire squamocolumnar junction of the transformation zone. The brush was then inserted into the vial containing preservative fluid. Liquid-based cytology system (Liqui- PREP by LGM International, Inc, Melbourne, FL, USA) for collection and transport of cervical specimen was used. The specimens were stored at -20°C for further analysis[7] (Mbamara *et al.,* 2011).

DNA Extraction:

Bioneer Accuprep Genomic DNA Extraction Kit (K-3032) was used for the extraction of the HPV DNA as follows:

Proteinase K was mixed with 1,250µl of deionized water and then 20µl of the solution was pipette into each labeled ependoff tube. Then 200µl of each sample was added to the tubes containing Proteinase K and then 200µl of GB (Binding) buffer was added to the mixture. It was mixed thoroughly. It was then incubated at 60 °C for 10 minutes. Then 400µl of ethanol was added and mixed. The lysate was carefully dispensed into the upper reservoir of the binding column tube (which is fit in a collection tube). It was then closed and centrifuged at 8000 rpm for 1 minute. The solution from the collection tube was then discarded and the collection tube placed back under the binding column tube for further analysis. Exactly 500µl of washing (WA) 1 buffer was added; the tube was closed and then centrifuged at 8000 rpm for 1 minute. The solution from the collection tube was discarded so as to reuse the collection tube again. Then 500µl of washing buffer (W2) buffer was added, the tubes were closed and centrifuged at 8000 rpm for another 1 minute and the solution from the collection tube was discarded. This was centrifuged once more at 13000 rpm for 1 minute to completely remove ethanol and droplet clinging to the bottom of the binding column tubes. The binding column tubes were transferred to a new ependoff tubes for elution. Fifty microlitre of EA buffer was added to the binding column tubes and kept for 1 minute at room temperature after which the mixture was centrifuged at 8000 rpm for 1minutes to elute into the ependoff tubes. The eluted DNA samples were then stored at -20 °C for amplification [8] (Bioneer.us.com, 2018).

Polymerase Chain Reaction:

All specimens were amplified HPV DNA using a nested Polymerase Chain Reaction (nPCR) with PGMY 09/11 primer for the first round and GP5+/GP6+ primer for the second round which amplifies a 450 bp and 150 bp fragment of the L1 HPV genomic region respectively (Gravitt *et al.,* 2000). Accu- Power HotStart Premix K-5051 (Bioneer Corporation, USA) was used for the PCR and Thermal cycler (Bio Rad) was used for PCR. The PCR kit contained PCR premix solution for a 20µl reaction.

Primers used were PGMY09 and PGMY11:

PGMY11-A- GCACAGGGACATAACAATGG

PGMY11-B- GCGCAGGGCCACAATAATGG

PGMY11-C- GCACAGGGACATAATAATGG

PGMY11-D- GCCCAGGGCCACAACAATGG

PGMY11-E- GCTCAGGGTTTAAACAATGG

PGMY09-F- CGTCCCAAAGGAAACTGATC

PGMY09-G- CGACCTAAAGGAAACTGATC

PGMY09-H- CGTCCAAAAGGAAACTGATC

PGMY09-Ia- GCCAAGGGGAAACTGATC

PGMY09-J- CGTCCCAAAGGATACTGATC

PGMY09-K- CGTCCAAGGGGATACTGATC

PGMY09-L- CGACCTAAAGGGAATTGATC

PGMY09-M- CGACCTAGTGGAAATTGATC

PGMY09-N- CGACCAAGGGGATATTGATC

PGMY09-Pa- GCCCAACGGAAACTGATC

PGMY09-Q- CGACCCAAGGGAAACTGGTC

PGMY09-R- CGTCCTAAAGGAAACTGGTC

HMB01b- GCGACCCAATGCAAATTGGT

Gp5+: 5′TTTGTTACTGTGGTAGATACTAC3′

Gp6+: 5′GAAAAATAAACTGTAAATCATATTC3′

[8] (Bioneer.us.com, 2018).

Sixteen microliters (16µl) of deionized water was added to each tube containing Hotstart PCR Premix while 18µl of deionized water was be added to the negative control tube. Then 2µl of the PGMY primer was added to each tube followed by 2µl of DNA template. This was then mixed to ensure that every component settles at the bottom of the tube. This was inserted into the thermal cycler to run for 35 cycles under the following conditions: Pre denaturing at 94°C for 5 minutes, Denaturing at 94°C for 1 minute, Annealing at 45°C for 1 minute, Extension at 72°C for 1 minute and final Extension at 72°C for 5 minutes. Upon completion, a second round was initiated using the end product of the first round. The process involved; sixteen microlitres of deionized water were added into a fresh hotstart premix, 2µl GP5+/6+ primer and 2µl of the first round end product. This was mixed and inserted into the thermal cycler to run for 30 cycles using the following condition: Pre denaturing at 94°C for 5 minutes, denaturing at 94°C for 30 seconds, Annealing at 40°C for 30 seconds, extension at 72°C for 30 seconds and final extension

at 72°C for 5 minutes. The final product was stored for further analysis. (Bioneer.us.com, 2018).

Products of HPV amplification were visualized using 1.5% agarose gel stained with ethidium bromide. This was then exposed to UV light with a gel documentation system. The DNA bands were visualized, using a gel imaging system (Gel Doc 2000, Bio-Rad Laboratories Inc., Hercules, CA, USA). The result was determined by extrapolating from the amplicon band and the ladder band.

HPV Sequencing Procedure:

Sequencing machine (Beckman Coultier CEQ 2000XL). Genotyping was achieved by direct sequencing using the Gp 6+ oligoprimer. Sequencing was done using the Terminator version 3.1 cycle sequencing kit reaction (Applied Biosystems, Foster City, California, USA). The sequencer used was ABI PRISM 310 genetic analyzer by Applied Biosytems, Foster City, California, USA; Model 310.

RESULTS

A total of fifteen samples were successfully sequenced and 9 HPV types isolated which includes HPV- 6, 16, 18, 31, 58, 66, 70, 72 and 81as shown in table 1.About 4 high risk HPV Type 16, 18, 31 and 58 were detected, HPV 66 which is categorised as probable carcinogenic was also identified and other HPV genotypes 6, 70, 72 and 81 were also identified under the low risk HPV Types.

The HPV DNA was detected in cervical swabs of women using consensus primer set MY09/MY11 and $GP5^{+}/GP6^{+}$. The result of HPV sequencing included High Risk HPV types such as HPV16 (13.33%), HPV18 (13.33%), HPV 31 (6.67%) and HPV 58 (13.33%) and HPV 66 which fall within the probable high risk had a prevalence of 6.67% while other types identified are characterised under the Low Risk HPV types and they included: HPV 6 (6.67%), HPV 70 (26.67%), HPV 72 (6.67%) and HPV 81 (6.67%). Among all the HPV types isolated, the most prevalent type was HPV 70 which had a prevalence rate of 26.67% (Table 2).

DISCUSSION

A total of 9 HPV types (HPV- 6, 16, 18, 31, 58, 66, 70, 72, 81) were successfully detected in 15 samples successfully sequenced indicating a circulation of HPV types in Abuja Nigeria, giving a frequency of 27.27% (15/55). The frequency of HPV Types (27.27%) in this study is higher than the study in Abuja by Akarolo- Anthony *et al.* [9] who had a frequency of 25%. A record of low rate of infection might be due to the fact that the study was restricted within HIV infected women while this research captured the general population of women. But studies have however shown that HIV positive women are prone to persistent HPV infection when the immune system has been compromised [11]. However, there was lower prevalence of HPV DNA positivity in a study from the southwestern Nigeria by Nejo *et al.,* [12] who recorded a rate of 17.3% and 23.7% as recorded by Dareng *et al.,* [13] among HIV positive women using the Roche Linear Array genotyping test.Ogah *et al.,*[14]also recorded a rate of 11.5% among women who had undergone Female genital Mutilation using the Gene flow through hybridization method.

Other studies done internationally also recorded a low prevalence rate of 6.5% as reported by Ayse *et al.,*[15] among Turkish women using the consensus primers as done in this study. Aziz *et al.* also recorded a rate of 4.7% among Pakistani women and the reason for such low prevalence rate could be as a result of the customary monogamous behaviour within the society of Pakistan [16]. Bedoya- Pilozo *et al.,* [17], however recorded a high prevalence rate of 68.1% among women in Ecuador.

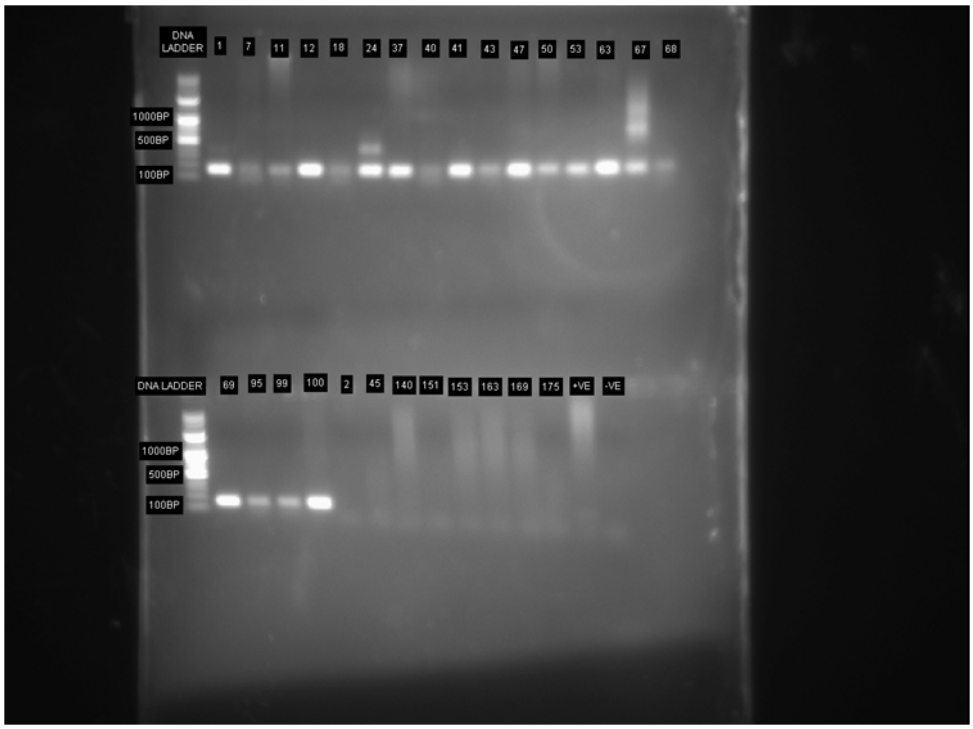

Fig. 1 : Detection of HPV isolate by Gel electrophoresis using 1.5% Agarose Gel Stained With Ethidium Bromide. The first lane had the ladder followed by samples tested and lastly the negative (-ve; nuclease free water) and Positive (+ve) control

Table 1 : HPV types isolated

Serial Number	Sample Number	HPV Type isolated
1	*1*	HPV- 18
2	*12*	HPV- 70
3	*24*	HPV- 18
4	*37*	HPV- 6
5	*47*	HPV- 72
6	*48*	HPV- 31
7	*63*	HPV- 70
8	*67*	HPV- 81
9	*100*	HPV- 16
10	*293*	HPV- 16
11	*332*	HPV- 70
12	*351*	HPV- 58
13	*365*	HPV- 58
14	*381*	HPV- 70
15	*394*	HPV- 66

Table 2 : Prevalence of HPV types identified

HPV Type	Frequency (%)
HPV 6	1 (6.67)
HPV 16	2 (13.33)
HPV 18	2 (13.33)
HPV 31	1 (6.67)
HPV 58	2 (13.33)
HPV 70	4 (26.67)
HPV 72	1 (6.67)
HPV 81	1 (6.67)
HPV 66	1 (6.67)

This study utilized nested PCR using PGMY09/ PGMY11 and GP5+/ GP6+ which has been shown to be sensitive in detecting wide range of HPV types [18]. The HPV types detected in this study were 6, 16, 18, 58, 70, 72 and 81 among which Type 16, 18 and 58 are classified as High-risk HPV while Type 6, 70, 72 and 81 are classified as Low-risk HPV. A study by Nejo *et al.* [12] recorded a prevalence rate of HPV type to be 17.3% in Oyo Southwestern, Nigeria. The predominant type in the study was HPV-31 similar to a study carried out in Lagos among HIV positive women which had the predominant HPV type to be HPV-31 [19]. Both studies indicated high prevalence among high risk types detected compared with this study that had a high prevalence among Low risk HPV types. This may be due to the geographic location of study area as high risk HPV have been recorded by [20, 19, 5, 21]. This study is also in agreement with studies by Aziz *et al.,* [16] who also reported a high prevalence among Low Risk HPV types.

The high risk types found were HPV 16, 18 and 58. This is similar to a finding in West African region with type 16 and 18 been the most common types isolated[22]. Bedoya- Pilozo *et al*[17],also identified high risk HPV- 16 and 58 as the most common type isolated among women in Ecuador. Studies among Sub- Saharan Africa women showed that HPV- 16, 18, 33, 35, 45 and 52 are the most commonly detected types [23] and also responsible for 80% cancers [24]. The predominant HPV type isolated in this study was HPV 70 with a prevalence rate of 33.33% which is contrary to a study a by Akarolo *et al.* [10], which recorded HPV 35 as the most prevalent. Nejo *et al.*[12], recorded HPV 31 as the most prevalent. However a study in Pakistan by Aziz *et al.* [16], also recorded a low risk HPV- 6 as the predominant HPV type detected. The prevalence of high risk HPV types among HIV positive women was 37.5% and isolated type 31, 35, 52 and 53 [19]. HPV Type 18 (44.7%) and 16 (13.2%) were the most predominant recorded by Manga *et al.* [25], and the same HPV Types were also predominant in the study carried out in Kano [26]. This might imply that there is a persistent of same HPV Types within the Northern geographic region of Nigeria.

The percentage prevalence of all the high risk HPV Type 16, 18 and 58 was 13.33% this is close to a study by Kolawole *et al.* [6], who recorded a prevalence of 20% for HPV 16 and 10% for HPV 18 respectively. A study by Thomas *et al.* [20], also reported a high frequency of HPV 16 and 35 was the most common, followed by HPV 31, 58 and 56. Similarly studies in Ghana and South Africa revealed that types 16, 18, 35 and 45 were the most common types in sub Saharan African women [23]. A prevalence of 17% of high risk HPV types was recorded Serwadda *et al* [27], in Uganda who used the Hybrid Capture (HC) assay II method in detecting HPV.

This study identified a woman with genital warts who had low risk HPV- 81 and this is similar to a study by Nejo *et al.*[12], who reported same. This implies that low risk HPV may be associated with genital warts and so relying on presence or visibility of warts alone is not enough as adequate HPV testing will be required to identify the type.

This study is similar to a research by Castellsague *et al.*[28], who reported HPV 35 been more common than HPV 16 among Mozambique women but also had HPV 16, 18 and 58 as the high risk type identified. A study in Senegal by Xi *et al.*[29], having HPV type 16, 18 and 58 as the high risk type identified was also reported, a study in Kenya recorded HPV 52 as the most common type found [30]. This study has significance as various studied have indicated the role of HPV 16 and 18 in cervical cancer development, this then suggest that there should be strong advocacy for the use of HPV DNA typing technique in routine diagnosis of HPV infection and cervical cancer. Also the isolation of high risk HPV among healthy women has reiterated the fact that in most cases, there is almost no clinical manifestation of HPV infection in the individual therefore the need also for routine HPV screening.

CONCLUSION

About 9 different HPV types were detected with the most predominant type being HPV 70. Other types identified were HPV 6, 16, 18, 58, 72 and 81. There is therefore a need to increase the level of surveillance on females at risk of cervical cancer in this environment, since significant proportion of highly oncogenic strains with a high tendency to transform into malignancy were observed in this study. There is also a need to encourage Government agencies to promote a more inclusive HPV vaccine in their national immunization scheme which has a wider coverage as against the one currently available in the country. This should be administered to girls at the appropriate age if HPV transmission is to be reduced. There is also need for sexual behaviour education and awareness in order to reduce the impact of the risks factors identified in this study. Population based study need to be done for further characterization of HPV genotypes in our environment.

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